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Mitophagy in Huntington's Disease
Mitofagie u Huntingtonovy choroby

Bachelor's thesis

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce a ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

Mitochondrial dysfunctions contribute to the progression of many neurodegenerative diseases, including Huntington's disease (HD). In HD, mutation in the huntingtin gene (HTT) results in the expansion of CAG repeats, causing the growth of the polyglutamine tract. This growth is responsible for the gain of toxicity function of the protein. The turnover of dysfunctional and damaged mitochondria is mediated via mitophagy – a selective form of autophagy. Additionally, mitophagy impairments have recently been described to play a key role not only in neurodegenerative diseases. The protrusion of mitophagy results in the clustering of defective mitochondria, organelles which are responsible for fulfilling the energetic demands of neural cells. The most distinctive impact of the impairment is on the striatal medium spiny neurons and results in the development of motor and cognitive dysfunctions. This thesis describes how HD affects mitophagy and reveals the biggest obstacle of mitophagy – disruption of mitochondria targeting into emerging autophagosomes caused by the abnormal interaction of mHTT and p62. Induction of mitophagy at this stage could be crucial for the future therapeutic research of HD. Generally, initiation of mitophagy could become a relevant therapeutic target for many other neurodegenerative diseases.

Keywords: mitophagy, autophagy, PINK1, neurodegenerative diseases, Huntington's disease

Abstrakt

Mitochondriální dysfunkce výrazně přispívají k projevu mnoha neurodegenerativních onemocnění, jako je například Huntingtonova choroba (HD). U HD, mutace genu pro huntingtin (HTT), kde dochází k expansi CAG repetic, způsobuje nárůst polyglutaminového řetězce, který je zodpovědný za toxicitu proteinu. Obrat nefunkčních nebo jinak poškozených mitochondrií je zajištěn skrze proces selektivní autofagie, mitofagie. Poruchy mitofagie byly recentně popsány jako klíčové u řady onemocnění nejen neurodegenerativního charakteru. Vedou k postupnému hromadění nefunkčních mitochondrií, organel zodpovědných za splnění vysokých energetických nároků neuronálních buněk. Tato neurodegenerace je nejvíce výrazná u „medium spiny" neuronů striatu a vede k vývoji motorických a kognitivních poruch. Tato práce shrnuje, jak HD ovlivňuje mitofagii. Dále odhaluje, že největším blokem mitofagie je nejspíše narušené cílení mitochondrií do formujících se autofagosomů, způsobené abnormální interakcí mHTT a ubiquitin vazebného proteinu, p62. Právě indukce mitofagie v tomto kroku by mohla být zásadní pro budoucí terapeutický výzkum HD. Iniciace mitofagie obecně by se pak mohla stát relevantním terapeutickým cílem ve vývoji léčby dalších neurodegenerativních onemocnění.

Klíčová slova: mitofagie, autofagie, PINK1, neurodegenerativní poruchy, Huntingtonova choroba

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Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
acetyl CoA	Acetyl coenzyme A
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
Atg	Autophagy-related
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
bp	Base pair
CAG	Cytosine-adenosine-guanin
cDNA	Complementary DNA
Cvt	Cytoplasm to vacuole targeting
Da	Dalton
Dnm1	Dynamine 1 gene
Drp1	Dynamine related protein 1
DRPLA	Dentatorubralpallidoluysian atrophy
e^{-}	Electron
ECFP	Enhanced cyan fluorescent protein
EM	Electron microscopy
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FIP 2000	Focal adhesion kinase-family protein of 200 kDa
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HD	Huntington's disease
hiPSCs	Human induced pluripotent stem cells
HOPS	Homotypic fusion and protein sorting
IA	Ibotenic acid
IMM	Inner mitochondrial membrane
KA	Kainic acid
Keap 1	Kelch-like ECH-associated protein 1
KTP	N^{6} furfuryl ATP
LC3	Microtubule-associated protein 1 light chain 3

LIR	LC3 interactive region
MARF	Mitochondrial Assembly Regulatory Factor
MEF	Mouse embryonal fibroblast
Mfn 1 and 2	Mitofusin 1 and 2
mHTT/HTT	Mutated huntingtin/huntingtin
MIRO	Mitochondrial Rho GTPase
MPTP	Mitochondrial permeability transition pore
m-RNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTS	Mitochondria targeting sequence
NADH	Nicotinamide adenine dinucleotide (reduced form)
nDNA	Nuclear DNA
NMDA	N-methyl-D-aspartate
Nrf2	Nuclear factor erythroid 2-related factor 2
OE	Overexpression
OMM	Outer mitochondrial membrane
PAS	Pre-autophagosomal structure
PBMCs	Peripheral blood mononuclear cells
PCr	Phosphocreatine
PD	Parkinson's disease
PE	Phosphatidylethanolamine
Pi	Organic phosphate
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PINK1	PTEN-induced putative kinase 1
poly P	PolyProline
poly Q	PolyGlutamine
prAPE1	Precursor of aminopeptidase 1
QA	Quinolic acid
r-RNA	Ribosomal RNA
SBMA	Spinorubral muscular atrophy
SCA	Spinocerebellar ataxia
Ser 65	Serin on the 65^{th} position of a certain protein
SIRT1	Sirtuin 1
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SQSTM1	Sequestosome 1

TFC	Total functional capacity
TIM	Translocase of inner membrane
TOM	Translocase of outer membrane
TOR	Target of rapamycin
t-RNA	Transfer RNA
UBA	Ubiquitin-associated domain
UBL	Ubiquitin-like domain
uESCs	Human embryonal stem cells
ULK 1 and 2	Unc-51-like kinase 1 and 2
VDAC	Voltage-dependent anion-selective channel 1
YAC	Yeast artificial chromosome

Introduction

Huntington's disease (HD) is an autosomal neurodegenerative disease caused by the CAG triplet expansion in the exon 1 of the *IT15* gene. The CAG triplet repeats in the mutated *IT15* gene encode an extended polyglutamine stretch which is known to be responsible for the gain of the toxicity of the mutated huntingtin protein (mHTT). Accumulation of the mHTT aggregates leads to the development of cognitive and motor dysfunctions, as the most distinctive impact occurs in neural tissue. So far, the exact cause of the pathophysiology of HD has not been revealed and remains to be determined.

Neural tissue needs to maintain a steady state of energy metabolism, as its energetical demands are extremely high. Common mitochondrial defects related to this disease result in an impaired energetic metabolism, making mitochondrial quality control crucial to the viability of neural cells. However, the process of mitochondrial quality control, mitophagy, is impaired as well. It thus makes mitophagy a relevant therapeutic target.

The aim of this thesis is to summarize current knowledge about the role of mitochondria in HD the contribution of mitochondrial defects to the pathogenesis of HD. The thesis describes the process of autophagy, its individual steps, and members of the autophagical cascade. It emphasizes the impact of impaired mitophagy and presents hitherto accomplished advances in the research of specific mitophagical aspects in the context of HD, including therapeutically relevant targets and possible approaches in the study of mitophagy.

1 Mitochondria

1.1 Structure and Function

The mitochondrion is an organelle surrounded by two membranes that have its own DNA, which means that we consider it a semiautonomous organelle. While the outer membrane is smooth, the inner one infolds and forms structures called *cristae mitochondriales*, which greatly enlarge the area of the membrane (Ernster and Schatz, 1981). Together they compartmentalize the mitochondrion into two different parts: the matrix and the intermembrane space (Frey et al., 2002). Both the outer and inner mitochondrial membrane consists of different types of protein. The most of these is encoded by nuclear DNA and then transported into the mitochondrion thanks to its translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM). The OMM needs to remain permeable, which is mainly secured by the presence of aquaporins. The IMM is much less permeable and comprises the electron-transport chain enzyme complexes. These enzyme complexes are required for the production of ATP through the process of *oxidative phosphorylation*. Electrons, transported to the *respiratory chain* by the NADH and FADH2 enzymes, are produced by converting pyruvate and fatty acids into acetyl CoA in the *Krebs cycle*. After that the transport of electrons begins – NADH is disassembled into NAD^+ and hydride ion, which is then converted into one proton and two electrons. These electrons pass through four respiratory enzyme complexes (Complex I–IV) that contain redox centres. Three of these enzymes maintain energy by pumping protons through the membrane. However, Complex II – succinate:ubiquinone reductase – does not and only acts as a vehicle for the electrons. The first and biggest complex of the respiratory chain is called NADH dehydrogenase complex (Complex I). It transfers electrons through ubiquinone to the cytochrome b-c_1 complex (Complex III), which is functional when it is dimerized. The dimer contains two active centres, the first one for oxidizing ubiquinol and the second one for reduction of ubiquinone. These two centres are needed to transport protons across the membrane and also to pass electrons to cytochrome c_1, which is actually the substrate for the Complex IV, the cytochrome oxidase complex. The complex is also a dimer and contains cytochromes and copper atoms essential for transport of the electrons to the last recipient, oxygen (Saraste, 1999). Finally, F_0F_1-ATP synthase (Complex V) is composed of two subunits: F_0 – rotor and F_1 – stator. The rotation is needed for producing ATP and here the energy conserved in proton gradient is finally used to synthetize ATP from ADP + Pi (Junge and Nelson, 2015).

1.2 Mitochondrial Genome

It is assumed that mitochondria arose from a photosynthetic prokaryotic organism – cyanobacteria (α-purple bacteria) (Blackstone, 1995). According to Lynn Margulis's theory of endosymbiosis, the bacteria, which was aerobic (the final recipient for the energy changeover was O_2), became a symbiote of an anaerobic organism (Margulis Lynn, 1981).

Thanks to that mitochondria contain their own DNA. Mammalian mitochondrial genome consists of 1,000–10,000 circular double-strand DNA molecules of about 16.5 kpb in length and the mass of 10^7 Daltons (Giles et al., 1980). In most protozoans, mtDNA encodes 37 genes, of which 22 are t-RNAs, 13 are m-RNAs, and 2 are r-RNAs (Wilson et al., 1985).

In most animals and also in humans the mitochondrial genetic material is inherited maternally. Even though the paternal mitochondria enter the ovum, the quantity is too low and it never really enters the next generation of offspring. Also the recombination is absent and is different in comparison to nuclear genes (Sato and Sato, 2013). The next thing which was observed and is not the same as in nucleus is faster evolution. This may be caused by higher mutation pressure and inefficient repair, as well as the fact that proteins editing mtDNA are not encoded in there (Ladoukakis and Zouros, 2017).

1.3 Mitochondrial Dynamics

Although mitochondria are often depicted as a single static oval shaped organelle, they appear to be highly dynamic. They continuously move in two highly coordinated processes – fusion and fission – in order to fulfil physiological requirements of the cell. It is important for keeping the mitochondrial network in a good condition and as it is important for maintenance of mitochondria, it also plays a big role in cellular processes like apoptosis and mitophagy.

Although morphological changes of mitochondria were already observed earlier, their closer examination was done many decades later with the use of fluorescent microscopy (Rizzuto et al., 1996). Mitochondria cannot originate de novo and thereby divide by the process of fission before cytokinesis. It is mainly the dynamin GTPase family proteins that are responsible for both the fusion and the fission processes. In yeast, the dynamin homologue responsible for membrane division during fission is Dnm1 (dynamin 1 gene), whereas in mammals it is Drp1 (dynamin-related protein 1) (Bleazard et al., 2002; Smirnova et al., 2001). The fusion of OMM is arranged by other Dynamin family members. The first evidence was provided by studying *Drosophila* sperm cells, where mutation in this protein located in OMM led to imperfect formation of nebenkern, a spherical structure formed by layers of concentrically organised mitochondria. This protein was named fuzzy onions because of the fuzzy onion-like appearance of mitochondria that were unable to fuse (Hales and Fuller, 1997). It has homologs in yeast (Fzo1p) and mitofusin 1 and 2 (Mfn1 and Mfn2) in mammals. Another fusion-associated protein, Mitochondrial Assembly Regulatory Factor (MARF), shares similar function with mitofusins (Dorn et al., 2011). The fusion of IMM is provided by Mgm1p in yeast and OPA1 in mammals, both dynamin-like GTPases (Belenguer et al., 2002; Wong et al., 2000).

2 Mitochondria and Neurodegeneration

2.1 Huntington's Disease

Huntington's disease (HD) is a neurodegenerative autosomal-dominant disease that is caused by elongation of the CAG repeats in the first exon of the *IT15* gene, which encodes the huntingtin protein (HTT). This leads to the growth of the polyglutamine strand at the N-terminus of the HTT and the protein thus gains toxicity. The age at which the disease can begin to manifest can be correlated with length of the polyglutamine stretch, i.e. a higher number of repeats means earlier onset of the disease (Trottier et al., 1995). It mainly affects middle-aged people, which can be a problem as is hereditary and the patients can already have children (Walker, 2007).

There are several other diseases where polyglutamine tract in a certain protein is responsible for their outbreak. Mentioned diseases are e.g. dentatorubralpallidoluysian atrophy (DRPLA), spinorubral muscular atrophy (SBMA), and spinocerebellar ataxia (SCA) (Li and Li, 2004).
Disorders can be observed when the threshold of 35-39 CAG codons is crossed. Patients with 40 or more repeats usually have the disease manifested with all the mitochondria associated symptoms that are typically observed in HD. These symptoms are: imbalance of energy metabolism in brain and peripheral tissues, lowered activity of electron-transport chain complexes II and III in striatal cells, and decreased membrane potential in mitochondria of lymphoblasts. The depolarization was higher as the number of CAG repeats was growing. The clinical phenotype can be distinct. It includes chorea and dystonia, incoordination, cognitive decline, and behavioural difficulties, e.g. manic and psychotic syndromes or depressions (Vonsattel and Difiglia, 1998).

Cells, in which the biggest damage (i.e. atrophy and loss) occurs, are those in striatum. Corpus striatum accompanied by amygdaloid nucleus is part of the basal ganglia. It also often includes the subtalamic nucleus and substantia nigra thanks to their linkage with the other parts of striatum. The corpus striatum can be divided into neostriatum (*caudate nucleus*) and paleostriatum (*globus pallidus*). Globus pallidus further includes external and internal segments. Striatal neurons send axons to the globus pallidus and the substantia nigra. In somatic tissues, mutant huntingtin undergoes ubiquitination (Vonsattel and Difiglia, 1998).

2.2 Huntingtin Protein

Wild-type HTT with the polyglutamine stretch of normal length (6-35 CAG repeats) is a large protein of about 350 kDa which also undergoes multiple posttranslational modifications. Throughout the cell HTT is mainly located in the cytoplasm of nerve cells, but it can also be found associated with many cellular compartments such as the nucleus, endoplasmic reticulum, Golgi complex, mitochondria, and endosomes.

HTT has an indisputable impact on endocytosis thanks to its interactions with clathrin, dynamin, and many other endocytosis-related trafficking proteins. By binding to Caspase 9, HTT prevents it from interacting with aptosomal complex and prevents the release of cytochrome C from mitochondria. It plays the role of a scaffold for molecular motor proteins involved in both anterograde and retrograde axonal transport and is thus also associated with the movement of mitochondria along axons of nerve cells. Defective axonal transport of mitochondria then causes lowered presence of energy supplies in places where energy is highly required such as synapses. mHTT supresses the expression of the PCGα-1 protein, which is responsible for the regulation of many mitochondrial proteins (Kolesnikova, 2013).

Although loss of the functions that HTT normally provides certainly affects HD pathogenesis, it is generally believed that the real source of the disease is the "gain of toxicity" by the growth of polyQ tract. This was proved by the evidence that polyQ prepared independently of the huntingtin exon 1 location or as a part of an unrelated protein could still stretch and led to the development of neurological symptoms in mice(Ikeda et al., 1996; Mangiarini et al., 1996; Ordway et al., 1997). HD along with other neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and prion diseases shows the presence of amyloid-like fibrils and the formation of inclusion bodies that compose of fibers of misfolded proteins with a β-sheet conformation (Perutz et al., 1994; Turmaine et al., 1997). There is a correlation between the length of the polyQ tract and the density of inclusions, which together with other experimental evidence suggests that this may play a key role in the pathogenesis of HD (Becher et al., 1998). Other studies showed that the degree of severity of the diseases did not only depend on the length of the polyQ, but also on the age of the testing, linking it to the rate of aggregate formation *in vitro*, i.e. the longer the polyQ repeat is, the faster the aggregation is. According to these observations, nucleation, which is closely connected to the concentration and length of the poly Q, was proposed to be the rate-limiting step in the aggregation, determining it to be the next possible therapeutic target (Heiser et al., 2002).

In order to answer the question how the aggregation can be inhibited or at least slowed down, Kenkichi's research group set and proved a new hypothesis that flanking polyQ proteins with other amino acid sequences could inhibit the aggregation by preventing it to form the nucleus (Nozaki et al., 2001). An example could be the polyproline (polyP) repeat in the HTT protein, which inhibits fibril formation of pathological-length polyQ in vitro, but only when located on the C-terminal end of the polyQ repeat and when it is at least six proline residues long (Thiagarajan et al., 2005).

2.3 Laboratory Models for Studying HD

When studying neurodegenerative diseases, model organisms become remarkably useful. The affected tissue is often hardly accessible and we have to obtain that tissue *post mortem*. There are numerous model organisms important for the advance in understanding HD, including various types such as excitotoxic lesion models, cellular models, and animal models. HD was stated as caused by a single

gene mutation along with several other neurodegenerative diseases like AD or ALS. That provides us with the opportunity to easily prepare transgenic animal models.

2.3.1 Excitotoxic Lesion Models

While transgenic animal models represent the possibility to study the molecular basis of mHTT's impact, they do not often show the most coherent neuropathological symptoms like striatal cell loss. The possibility to observe possible aftereffects of striatal atrophy is probably the biggest benefit of using excitotoxic lesion models. It is important for understanding the impact of cell loss as well as for studying therapeutic procedures aimed at reducing the atrophy or replacing lost neurons. Intrastriatal injection of one of the below mentioned neurotoxins leads to the activation of N-methyl-D-aspartate (NMDA) receptors, which are quite common in the striatum, and thereby leads to the excitotoxicity, which can result in cell death (Coyle and Puttfarcken, 1993).

The most common toxin used for this method, quinolinic acid (QA), is an NMDA receptor agonist that can representatively mimic the loss of medium spiny neurons with relative preservation of aspiny NADPHd (NADPHd) neurons, and an increase of concentration of somatostatin and neuropeptide Y similarly to HD (Beal et al., 1986). Other neurotoxins like kainic acid (KA) and ibotenic acid (IA) are also capable of acute striatal neurodegeneration. However, lesions caused by them were unlike those produced by QA because their impact also included somatostatin/neuropeptide Y neurons (Flint Beal and Martin, 1983). Furthermore, intrastriatal injections of mitochondrial toxins (i.e. malonate or 3-nitro propionic acid) into rat brains have been shown to be capable of imitating the effects of QA and NMDA, indicating that defective mitochondria also play a big role in the pathogenesis of HD (Greene et al., 1993).

2.3.2 Cellular Models

Although *in vivo* HD rodent models and other HD rodent-derived models such as primary cultures and others proved their crucial value in research, there is a high demand for the development of human-derived models, which would give researchers the insight into the consequences of mHTT expression in specific cell types among CNS. Human pluripotent stem cells (hPSCs) can be such an answer. Generating them from human embryonic stem cells (hESCs) is surrounded with ethical and political concerns. Another possibility is to derive human induced pluripotent cells (hiPSCs) from human somatic tissue.

The first hESC lines were obtained from an HD embryo which was selected during *in vitro* fertilization thanks to preimplantation genetic diagnoses (Rechitsky et al., 2005). As these hESCs were obstructed by ethical problems, the development of hiPSC started and in 2007 two studies presented hiPSC prepared through viral vectors carrying transcription factors considered pluripotency hallmarks such as OCT4, SOX2, NANOG, LIN 28, c-MYC and KLF4 (Takahashi and Yamanaka, 2006; Vodyanik et al.,

2007). The huge advantage of using hiPSCs is the opportunity to get them noninvasively from the majority of somatic cells using patient-derived tissues.

Recently, peripheral blood mononuclear cells (PBMCs) were described to have the potential for the study of HD and its impact. The main advantage is that they are easily obtainable without any big invasive procedures like surgical intervention. PBMCs exhibited lowered amounts of mitochondrial biogenesis related factors. As shown earlier, mtDNA is altered in response to modified mitochondrial metabolism (Pawar et al., 2018). There was an overall incline in downregulation of genes linked to DNA repair, suggesting that metabolic changes in HD PBMCs are not anything uncommon. Although there was no significant mtDNA damage, nuclear DNA (nDNA) damage was notably higher than in control cells. The results of comparison of nDNA damage and total functional capacity (TFC) score of HD as well as disease duration presented nDNA damage in PBMCS to be a plausible biomarker for studying HD (Askeland et al., 2018a).

2.3.3 Nonmammalian Models

In past years, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and more lately *Danio rerio* were engineered to express mutant version of huntingtin. Every one of them provides some specific features that make it suitable for this purpose. Although there is a huge evolutionary gap between nonmammals and mammals, the cellular pathology seems to be highly conserved in all of them. Especially *Drosophila melanogaster* and *Saccharomyces cerevisiae* proved to be pivotal subjects in the previous findings. *D. melanogaster* exhibits an architectonically similar nervous system with several area divisions specialized in vision, olfaction, learning and memory. Furthermore, approximately 70% of known genes linked to neurodegenerative diseases in humans were found to have a related gene in *D. melanogaster*. The reason why *S. cerevisiae* is such a popular model is that it is easily genetically manipulated, its genome is well studied, reproduction time is quite fast and also the essential biological processes are highly conserved in yeast.

Overall, also the similarity in drug effects is outstanding, although awareness is still in place (Reiter et al., 2001). A big contribution of nonmammalian model organisms to therapy development is target validation. This can be a problem when testing specific compounds *in vitro*, where it has a certain impact, but when tested *in vivo*, the compound can target a different protein and has no impact at all. In these model organisms, the original target of the particular compound can be removed by genetic manipulation showing us whether there is or is not another target with possible therapeutic relevance. Such an example could be resveratrol, a polyphenol phytocompound found in grapes, which is a sirtuin 1 (SIRT1; homolog of the *S. cerevisiae* Sir2 protein) activator (Parker et al., 2005). SIRT1 is an NAD-dependent p53 deacetylase (Vaziri et al., 2001) and is a member of the Silent Information Regulator family genes, which extend *S. cerevisiae* and *C. elegans* lifespan(Tissenbaum and Guarente, 2001; Zipkin et al., 2003). Resveratrol prevented neuronal dysfunction caused by 128 polyQ repeat in *C.*

elegans, but rescue provided by resveratrol was disrupted in sir-2.1 and daf-16 mutants. Regarding this fact, it is believed that the prevention of mutant polyQ pathogenesis was done through targeting sir-2.1 and daf-16 (Parker et al., 2005).

2.3.4 Animal Mammalian Models

The most common model amongst mammals is the rodent model. Today, there are more than 20 distinct rodent models of HD. The first transgenic mice models – R6/1 and R6/2 – were developed in 1996. They express exon 1 of human *IT15* gene with approximately 115 and 150 CAG repeats under control of the human huntingtin promoter (Mangiarini et al., 1996). After the discovery of the R6 mice model, many others have been designed, varying in the length of CAG expressed, size of the fragment, and also the type of promotor. For example, R6 mice express only exon 1 out of 67 exons in the entire gene. This means that they have only 3% of the N-terminus of the protein encoded, including the polyQ stretch. The knock-in mouse model of HD uses endogenous mouse promotor to produce human mHTT. The promotor controlling the gene influences degree of its expression and thus it also influences the pathology of HD. Mice carrying yeast artificial chromosome (YAC) were introduced in order to develop a mouse model displaying more significant behavioural symptoms with earlier onset. In contrast to R6 mice, YAC mice express a full-length human mHTT (Hodgson et al., 1999). Almost ten years later, the bacterial artificial chromosome (BAC) mouse model expressing full-length human mHTT was introduced (Gray et al., 2008). It showed symptoms and phenotype coherent with those exhibited in YAC mice such as cognitive dysfunction, motor deficit, and neurodegeneration in the cortex and striatum (Gray et al., 2008; Van Raamsdonk, 2005).

Despite their previous contribution like revealing nearly the most fundamental pathological hallmark, the formation of aggregates and inclusion bodies, rodent models are not the most suitable ones since the majority of them does not have progressive neurodegeneration similar to the one in human patients. Big animals represent a better opportunity to monitor more human-patient likely pathologic features. In 2001, Chan et al. demonstrated transgenic monkey (Chan et al., 2001), but it took another 7 years to develop a transgenic nonhuman primate model of HD. By implementing lentivirus into fertilized oocytes of *Makak rhesus,* researchers achieved the goal and successfully expressed 84Q long human mHTT under the control of human ubiquitin promotor. Although two of the five monkeys, rHD-4 and rHD-5, died perinatally, the other three new-borns survived, one (rHD-3) of which for only one month, but it was sufficient to develop choria and dystonia. Researchers observed rare involuntary movements in rHD-2 one week after its birth. rH1 with normal length polyQ tract did not develop any symptoms, as predicted (Yang et al., 2008).

After the creation of transgenic monkeys, scientists focused more on domesticated farm animals. Sheep do not have any special requirements for housing since they usually live in a herd on a pasture, which means that expenses are not high. They are also relatively long-lived and not as much inbred as some

other animals, making them suitable for this purpose. Longevity allows one to observe later phases of neurodegeneration. Mature sheep have a comparable weight to a human adult. Similar weight makes therapeutic testing easier, although bigger weight is related to bigger size, making manipulation with them more complicated. Bigger size together with similar physiology can also be a benefit, enabling sheep to carry transmitting devices constructed for humans. Furthermore, they are unable to remove these devices by themselves. Although all these factors are crucial, the most important aspect is their brain. The brain is big enough and anatomically more similar to the human brain than the mouse brain is. Therefore, the sheep brain is suitable for preclinical studies. First HD sheep were prepared by microinjections of a full-length human mHTT cDNA carrying 73 CAG repeats and human promotor region, resulting in the birth of six lambs. Although the lambs did not display any overt symptoms seen in human, immunohistochemical assays using medium sized spiny neuron marker, DARP-32, showed their decrease (Jacobsen et al., 2010). The only condition that was found in these presymptomatic sheep was metabolic disruption. (Bawden et al., 2016).

The pig has more or less the same advantages as sheep, but is even more suitable as an HD disease model. Its brain is similar to the sheep brain – not only structurally but size wise. Pigs live relatively long (12–15 years) and are physiologically more similar to the human, especially in terms of the gastro-intestinal system and metabolic regulation. They also have a short generation time of 1 year. After they reach sexual maturity at a relatively young age (5–8 months), they start reproducing and continue to do so throughout the whole season, producing many offspring at a time (10–12 piglets) (Aigner et al., 2010). The tibetian HD miniature pig was created by a pronuclear injection of human mHTT cDNA under the control of chicken beta-actin promoter with cytomegaloviral enhancer. Two transgenes (N208-105Q and N208-160Q) were prepared this way. Both of the transgenes were connected to an enhanced cyan fluorescent protein (ECFP) through the small viral peptide F2A that can be self-cleaved in mammalian cells, allowing separation of the ECFP. All N208-160Q embryos died prenatally, suggesting that 160 CAG repeats lead to high toxicity disabling early development. N208-105Q transfection led to a successful birth of five piglets. PCR analysis of their DNA demonstrated positive expression of N208-105Q transgene. However, 3 of the five piglets died within 3 days. The fourth piglet died 25 days after birth, leaving only one piglet alive. The 3 piglets had difficulties sucking milk and thereby had weight gaining complications. Examination of N208-105Q pig brains showed apoptotic neurons with fragmented nuclear DNA. Moreover, the pig that died third, having lived for 53 hours, displayed hyperkinesia similar to chorea seen in HD patients. The fact that 160Q pigs died prenatally, along with the fact that the pig that died at the age of 25 days had the second shortest polyQ repeat, and the only living pig had the shortest polyQ repeat provided yet another piece of evidence that the length of polyQ tract influences the severity of the disease (Yang et al., 2010). A new pig model called Liběchov pig came in 2013 and was developed at the Institute of Animal Physiology and Genetics in Liběchov, Czech Republic. Lentiviral vector used in this transgene expression was constructed using the first 588 aa of

human HTT cDNA with 145 CAG/CAA triplets linked to an HD promoter. This element, inserted into PHIV7 plasmid containing cPPT and WPRE cis-enhancers, was transiently co-transfected into HEK293T cells for further production. Microinjections of HIV1-HD-548aaHTT-145Q into perivitelline space of pronuclear stage embryos resulted in the birth of 6 offspring, of which only one carried the transgene. This transgene pig was successfully bred up to third generation and like the sheep it displayed no overt HD symptoms at the time of the publication, except reduced fertility in F1 generation. The number of spermatozoa was significantly reduced in the ejaculate of the HD pig. In addition, the capability to successfully penetrate the oocyte was lower (Baxa et al., 2013). Later it was hypothesized that the reduced penetration rate might be tightly linked to mitochondrial metabolism, as the penetration of *zona pellucida* has high demands on ATP production and the capability to penetrate was restored after the *zona pellucida* was enzymatically removed. However, there was no evidence that would directly show that the affected fertilization was a result of modified mitochondrial metabolism (Krizova et al., 2017). A recent study of 48-months-old HD minipigs described behavioural changes common to HD neuropathology. In contrast to HD minipigs of up to 40 months old, which had normal motor and cognitive functions, the HD minipigs at the age of 48 months showed significant locomotor disability when performing a tunnel test, suggesting the onset of neurological impairment (Askeland et al., 2018b).

2.4 Mitochondria and Huntington Disease

Although mitochondria are essential for energy maintenance and therefore life preservation, they are also important for mediating cell death through apoptosis. Cytochrome C released from the intermembrane space to the cytosol binds to Apaf1 and procaspase-9 to form an apoptosome. After the apoptosome formation, procaspase-9 changes to its active form, caspase-9, resulting in a caspase cascade that leads to cell death (Li et al., 1997). As neurons, which are the most affected cells in HD, have high energetic demands and display apoptosis, mitochondria are supposed to play the key role in their death.

There is much evidence underlying the fact that impairment of energy metabolism impacts pathogenesis of HD. Increased lactate levels in cerebral cortex and basal ganglia, measured by the nuclear magnetic resonance spectroscopy (^{1}H NMR), showed the defect of oxidative phosphorylation and were the first piece of evidence to prove it in vivo (Jenkins et al., 1993). Additionally, succinate oxidation assays of mitochondrial membranes obtained via brain autopsies displayed a striking decline in mitochondrial respiration, cytochrome oxidase activity, and levels of cytochrome aa_3, suggesting that Complex IV activity is highly diminished (Brennan et al., 1985). Complex II and III of the respiratory chain were also found to be defective (Browne et al., 1997). As mHTT expression is omnipresent, abnormal mitochondrial functions can be found within other tissues. Skeletal muscles exhibit lowered phosphocreatine (PCr) levels in resting muscles and lowered ATP:PCr + Pi ratio even in studied presymptomatic subjects (Lodi et al., 2000). Both symptomatic and presymptomatic HD subjects displayed reduced mitochondrial membrane potential, higher cytochrome c release and higher caspase activity (Ciammola et al., 2006). Mitochondrial ATP production defects were also described in HD

lymphoblasts. Similarly to brain cell mitochondria, mitochondria from lymphoblasts exhibit reduced mitochondrial potential and lower concentration of Ca^{2+} ions necessary for depolarization, suggesting damage in calcium homeostasis (Panov et al., 2002). Isolation by swelling–shrinking procedure and immunoblot analysis of mitochondrial compartments led to a finding that mHTT interacts with mitochondrial permeability transition pore (MPTP) in OMM, decreasing the Ca^{2+} concentration necessary for MPTP to open (Choo et al., 2004). Besides the straightforward impact on mitochondria, transport of mitochondria along cortical neurons is hindered due to the effect of mHTT (Chang et al., 2006).

3 Autophagy

Since Huntington's disease affects mitochondria and fragmented mitochondria are connected to the disease, a mechanism that would maintain a state without damaged mitochondria is required. Generally, the process that organisms use to maintain a state without damaged organelles, to recycle used proteins and also to get rid of invasive pathogens is called autophagy. Autophagy is a highly controlled process where the isolation membrane starts to bud from a formation site called the autophagophore assembly site or pre-autophagosomal structure (PAS) (Figure 1). In yeast, there is only one PAS, whereas in mammals there are multiple places that act as the formation (He and Klionsky, 2009; Youle and Narendra, 2011). The autophagophore further grows and engulfs cytoplasm and its components, forms a two-membrane structure called autophagosome and fuses with lysosome to produce basic nutrients like amino acids and saccharides. As there were found many homologs among higher eukaryotes thanks to genetic screening of autophagy-related genes (Atg), it has been suggested that autophagy is evolutionary highly conserved. Main Atg proteins are summarized in Table 1. Undergoing conditions such as nutrient deprivation, mainly random portions of cytosol are processed in the non-selective path of autophagy. Another pathway uses specific cell components as substrate and because of that it is called selective autophagy. An example of substrate specific autophagy is cytoplasm-to-vacuole targeting (Cvt) pathway, which is similar to the classic bulk autophagy except that it occurs under physiological conditions and is biosynthetic (Klionsky and Emr, 2000). To distinguish between these selective processes, names that belong to them are connected to the substrate that a given process requires e.g. mitochondria (mitophagy), ribosomes (ribophagy), peroxisomes (pexophagy), and endoplasmic reticulum (reticulophagy) (He and Klionsky, 2009; Yoshii and Mizushima, 2015). The above-mentioned type of autophagy could be referred as to as macrophagy. Not that much known process is microautophagy. It serves for the direct uptake of cellular components into lysosome and instead of protection during starvation its purpose is to regulate the size of organelles and membrane composition. While macroautophagy is Atg proteins dependent, it seems that microautophagy does not require them and is instead regulated by TOR (target of rapamycin) and EGO (Uttenweiler et al., 2006).

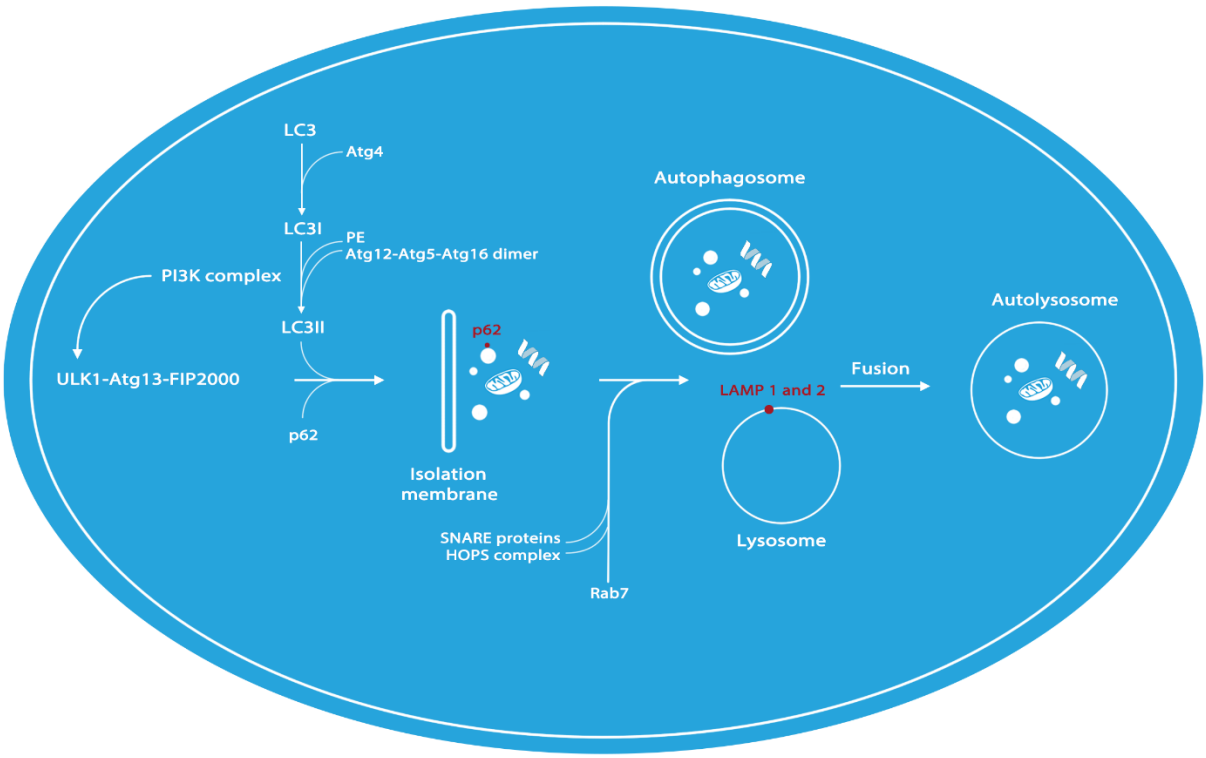

Figure 1: **A general diagram of autophagy in mammalian cells.** Adapted from (Ohmuraya et al., 2012). After recruiting the PI3K complex, along with other Atg proteins, to the ULK1-Atg13-FIP200, the isolation membrane starts to grow. LC3 is then processed by Atg4 and conjugated together with PE in presence of the Atg12-Atg5-Atg16 dimer. Accumulation of these factors at the autophagosome formation site is important for the growth and completion of the autophagosome. The cargo is labelled with the p62 and connected to the LC3-positive isolation membrane. After the autophagosome is complete, SNARE proteins, the HOPS complex and Rab7 enter the process to allow the fusion of the autophagosome and the LAMP flagged lysosome into the autolysosome. The sequestered cytoplasmic cargo is then degraded. PI3K - phosphatidylinositol 3-kinase; PE – phosphatidylethanolamine.

Table 1: **A summary of main Atg proteins involved in autophagosome formation**. Adapted from (Feng et al., 2014).

Yeast	Mammals	Characteristics/Function
Atg1	ULK1/2	Ser/Thr protein kinase; negatively regulated by mTOR; autophagy initiation
Atg4	Atg4A/B/C/D	Cysteine protease; Atg8 activation
Atg5	Atg5	Conjugated in the Atg12-Atg5-Atg16 complex; role in autophagosome formation
Atg6/Vps30	Beclin-1	Component of PI3K complex; regulated by Bcl-2
Atg8	LC3/I/II; GABARAP; GABARAPL1/2/3	Ubiquitin-like protein; conjugated to PE; important for autophagosome formation
Atg9	Atg9A/B	Transmembrane protein; possibly a shuttle
Atg11	-	Adaptor protein important for PAS organization in selective autophagy
Atg12	Atg12	Ubiquitin-like protein; conjugated in the Atg12-Atg5-Atg16 complex
Atg13	Atg13	Phosphorylated by mTOR; linker between Atg1 and Atg17; autophagy initiation
Atg14	Atg14(L)/Barkor	Component of PI3K complex
Atg15	Atg15	Intravacuolar disintegration
Atg16	Atg16L1	Conjugated in the Atg12-Atg5-Atg16 complex; role in autophagosome formation
Atg17	FIP200 (functional homolog)	Phosphorylated by ULK1; autophagy initiation
Atg18	WIP1/2/3/4	PI3P-binding protein
Atg19	-	Cargo recognition in selective autophagy
Atg20	Snx30	PI3P-binding protein
Atg21	-	PI3P-binding protein
Atg22	-	Breakdown of autophagic bodies
Atg24	Snx4	PI3P-binding protein
-	Atg101	Stabilizes Atg13 and ULK1

3.1 Mechanism of Autophagy

Autophagy is a highly complex mechanism that includes the following steps: induction of autophagy, cargo recognition, autophagosome formation, fusion of autophagosome with lysosome, cargo degradation, and efflux of products obtained from the degradation back to the cytosol. At the end, lysosome needs to be recycled and that is done mainly by the Golgi complex.

3.1.1 Induction of Autophagy

As autophagy emerges as adaptation to stress conditions, in the absence of stress factors it needs to be maintained on low levels by a certain mechanism. TOR is a serine-threonine protein kinase that represses Atg1 and thereby mediates autophagy inhibition. In yeast, after TOR is inactivated either by rapamycin or starvation, Atg1 associates with at least eight other Atg proteins of which Atg13 and Atg17 are the most important since they are included in the Atg1-Atg13-Atg17 scaffold and recruit other Atg proteins to organise PAS and therefore initiate autophagosome formation (Mizushima, 2010). Two homologs of Atg1 were identified in mammals, Unc-51-like kinase 1 (ULK1) and 2 (ULK2) (Ganley et al., 2009). The function of ULK2 is still not clear, but knockdown of ULK1 leads to an interruption of autophagy (Young, 2006). Focal adhesion kinase-family protein of 200 kDa (FIP200) is also considered as a homologous protein, although it has low sequence identity, but it shows structure and function as Atg17. These proteins form the ULKs-Atg13-FIP200 complex, which is again regulated by mTOR and initiates autophagy (Hara et al., 2008). In this case, Atg13 becomes phosphorylated by mTOR under nutrient rich conditions and thus blocks autophagy. Upon starvation, Atg13 is hypophosphorylated, binds ULKs and mediates phosphorylation of FIP200 done by ULKs. Not only do ULKs phosphorylate FIP200, they phosphorylate Atg13, but on different residues, which leads to an opposite effect, activation (Jung et al., 2009). Another component, Atg101, that was only found in mammals, stabilizes Atg13 and ULK1 and is essential for their phosphorylation (Hosokawa et al., 2009).

3.1.2 Cargo Recognition

In yeast Cvt pathway, the cargo, a precursor of aminopeptidase I (prApe1), includes a signal for targeting into a vacuole. The signal is recognized by Atg19, which then binds to the cargo. With the help of the adaptor protein Atg11, the Atg19-prApe1 complex is recruited to the PAS. At this point, Atg19 interacts with Atg8 and packages the cargo into Cvt vesicles (Lynch-Day and Klionsky, 2010). In eukaryotic cells, proteins can be degraded in proteasome (short-lived proteins) or by autophagy (longlived proteins). Degradation via autophagy is mediated through p62/sequestosome 1 (SQSTM1) in mammals and Ref(2)P in drosophila. The p62 binds ubiquitinated proteins to mammalian Atg8 homolog LC3 (microtubule-associated protein 1 light chain 3), which again acts as a autophagy effector (Bjørkøy et al., 2005). Another possibility of cargo recognition is pexophagy. In *P. pastoris,* ppAtg11 and ppAtg28 seem to be responsible for peroxisome recognition and targeting for degradation (Sakai et al., 2006).

3.1.3 Autophagosome Formation

Phosphatidylinositol 3-phosphate (PI3P) is a phospholipid found in cell membranes that is required for autophagosome formation. It is produced by the class III phosphatidylinositol 3-kinase (PI3K) complex, which composes of Vps34 (vacuolar protein sorting 34; PI3K activity), Atg14 (Atg14(L) or Barkor in mammals), Vps15 (p150 in mammals), and Atg6/Vps30 (Beclin-1 in mammals). The function of beclin-1 is regulated by Bcl-2 (B-cell lymphoma 2), which can inhibit autophagy by separating beclin-1 from the PI3K complex (Mizushima et al., 2011). Other yeast Atg proteins – Atg18, Atg20, Atg21, and Atg24

– bind to PI3K and further interact with two ubiquitin-like conjugation systems. Atg8 has multiple mammalian orthologs (Table 1.) that are part of the LC3 and GABARAP (gamma-aminobutyric acid receptor-associated protein) subfamilies. Upon starvation, i.e. conditions optimal for autophagy, most of the Atg8, after it is processed by a cysteine protease, Atg4, is conjugated to phosphatidylethanolamine (PE), due to the presence of the dimer of the second conjugation complex Atg12-Atg5-Atg16 (Nakatogawa et al., 2007). These complexes together with the above mentioned Atg proteins are then recruited to the PAS, which is essential for the regulation of the autophagosome growth (Matsushita et al., 2006; Nair et al., 2012).

The origin of autophagosomal membrane can be linked to more than one place. Mitochondria, ER and GA are considered to act as the possible origin, but what still has not been discovered is, how are the additional membranes translocated and then connected to the phagophore (Tooze and Yoshimori, 2010). Atg9 is the only conserved integral membrane Atg protein. In yeast, Atg9 cycles between peripheral sites, where the levels of most conserved Atg proteins are too low to be noticeable, and PAS. According to this observation, the hypothesis that Atg9 is part of the membrane delivery mechanism is supported (Mari et al., 2010; Nakatogawa et al., 2007).

3.1.4 Fusion of Autophagosome and Lysosome

The conjugated form of Atg8-PE is found on both sides of the isolation membrane and when the autophagosome formation is completed, Atg8 anchored to the outer membrane is dissociated from PE by the Atg4 and released to cytosol (Kirisako et al., 2000). This finalized autophagosome is now ready to fuse either with early or late endosome. In mammals, the fusion itself is provided by the small GTP binding protein Rab7 and the lysosomal-associated membrane protein 1 and 2 (LAMP-1 and 2) (Jager, 2004). The main components of the machinery in yeast are proteins that belong to the class C Vps/HOPS complex – the Rab7 homolog Ypt7; the SNARE proteins Vam3, Vam7, Vti1 and Ykt6; Sec17, Sec18 and Sec19 – homologs of NSF, SNAP and GDI; and Ccz1 and Mon1 (Klionsky, 2005).

3.1.5 Cargo Degradation

Components that were earlier consumed have to be degraded and then recycled, so they can be reused to replenish desired supplies. The vesicle breakdown is related to the acidic pH in the lysosome and also proteinase A and B encoded by the Prb1 and PEP4 gene (Takeshige et al., 1992). There are also two Atg proteins included in this last step – lipase Atg15 and Atg22. Unlike Atg15, Atg22 is only required for the breakdown of autophagic bodies (Klionsky, 2005).

4 Mitophagy

The quality control of mitochondria can be maintained by autophagy, which is very important not only during nutrient stress, but also plays an unquestionable housekeeping role in organelle turnover. Mitophagy has many key steps in common with the canonical macroautophagy pathway. Unlike the canonical macroautophagy pathway, mitophagy includes steps which are unique for the sequestration of mitochondria into autophagosomes and their further processing.

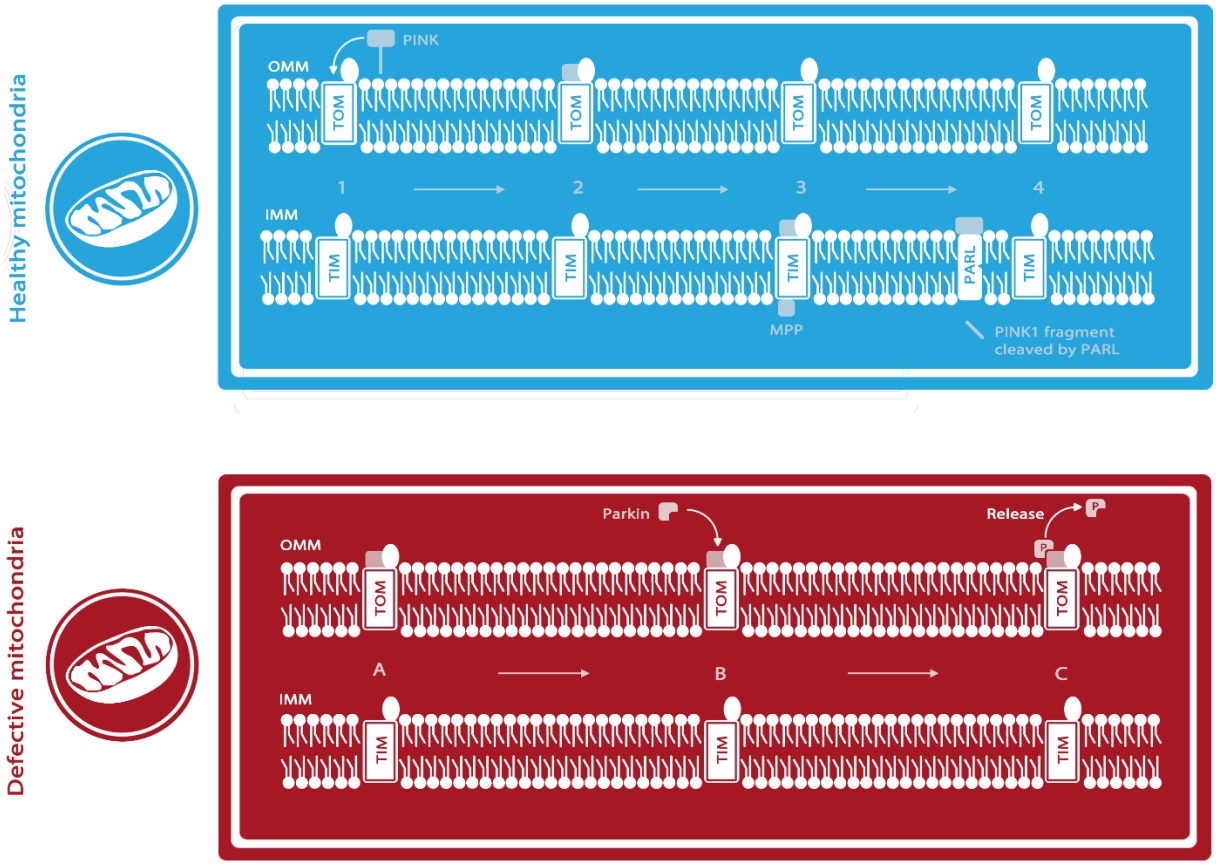

Figure 2: **Mechanism of mitophagy initiation**. Adapted from (Jin and Youle, 2012). Blue – healthy mitochondria: 1 – PINK1 is recruited to the TOM complex, 2 - PINK1 is translocated through the TOM complex, 3 – PINK1 is transported to the TIM complex and the PINK1 MTS is cleaved by MPP, 4 – PINK1 is processed by PARL and further degraded; Red – Defective mitochondria: A – PINK1 is transported to the TOM complex, B – Parkin is recruited to PINK1, C – PINK1 phosphorylates Parkin, which can now ubiquitinate proteins on OMM; TOM – translocase of outer membrane; TIM – translocase of inner membrane; MTS – mitochondrial targeting sequence; MPP – mitochondrial processing peptidase; OMM – outer mitochondrial membrane.

4.1.1 Incorporation of Damaged Mitochondria into Autophagosomes

The most studied pathway in mammalian tissues is probably the PINK1/Parkin pathway because the genes encoding PTEN-induced putative kinase 1 (PINK1) and an E3-ubiquitin ligase Parkin were

described as the cause of a recessive form of Parkinson's disease (Kitada et al., 1998; Valente et al., 2004). In healthy cells, the N-terminus of the PINK1 containing the mitochondrial targeting sequence (MTS) is translocated into the mitochondrial matrix and cleaved by the mitochondrial processing peptidase (MPP) (Figure 2) (Greene et al., 2012). Afterward, the rhomboid-like serine protease PARL cleaves the transmembrane domain and the rest of the protein with its exposed N-terminus is detached and degraded (Meissner et al., 2011). The decrease of mitochondrial membrane potential, which is typical of damaged mitochondria, results in PINK1 stabilization and recruitment of Parkin. PINK1 then phosphorylates parkin S65 in the ubiquitin-like (UBL) domain, which is responsible for the negative autoregulation of parkins E3 ligase activity. Another step to promote Parkin activation is phosphorylation of ubiquitin S65 similar to the one in the UBL domain (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). The phospho-ubiquitin allows Parkin to translocate to damaged mitochondria via the polyubiquitin chain linkage (Shiba-fukushima et al., 2014). Parkin accumulating at the OMM marks proteins with Parkin-dependent ubiquitination, allowing mitochondria to be further processed. The efficiency of mitophagy is at this point dependent on mitochondrial dynamics i.e. fusion and fission, as only fragmented mitochondria can be engulfed by the autophagosome. Thus the machinery regulating the dynamics is closely connected to mitophagy, and post-translational modifications of proteins important for mitochondrial fission and degradation of proteins ameliorating fusion of mitochondria are quite common, e.g. SUMOylation of Drp1 (Dynamine-related protein 1) (Campello et al., 2014). Parkin also directly prevents mitochondria from fusion. Mfn1 a 2 and Miro (Mitochondrial Rho GTPase) were identified as substrates of Parkin. Their ubiquitination leads to their degradation and thereby deterioration of fusion (Wei et al., 2015).

The sequestration of ubiquitinated mitochondria into the autophagic structures is mediated by the ubiquitin-binding adaptor proteins such as p62/SQSTM1, NBR1, and optineurin, and by the LC3/GABARAP family proteins, which also have the ubiquitin-binding domain in the LC3-interacting region (LIR) motif (Geisler et al., 2010). LC3/GABARAPs conjugate with PE. The conjugated form is associated with the isolation membrane and therefore can help with recruiting mitochondria (Pankiv et al., 2007).

4.1.2 Autophagosome-Lysosome Fusion

In mammals and *Drosophila*, mature autophagosomes fuse with lysosomes in a manner that involves the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNARE) syntaxin 17 (Stx17). Autophagosome associated Stx17 pairs with the R-SNAREs on late endosomal/lysosomal membrane creating the Stx17-VAMP7/8 trans-SNARE complex, which further interacts with Q_{bc} – SNARE SNAP29 (Itakura et al., 2012; Wang et al., 2016). A four-helix cluster formed by this SNARE complex plays a crucial role in fusion completion. Normally, it is being disassembled, but in order to accomplish the fusion the homotypic fusion and the protein sorting (HOPS) tethering complex stabilises the trans-SNARE bundle (Xu et al., 2010).

4.2 Mitophagy in HD

As mentioned above, mitochondrial defects, including dissipation of membrane potential and impairment of mitochondrial respiration, are one of the main hallmarks of HD (Bossy-Wetzel et al., 2008). Exceptionally high requirements on ATP supplies used for axonal transport, preservation of ion gradients, membrane potential and production of synaptic vesicles make neurons highly vulnerable to mitochondrial damage, since neurons primarily rely on oxidative phosphorylation as a source of ATP production. A process to maintain healthy mitochondria is required, especially in cells that are as vulnerable as neurons. However, even mitophagy, the process that should keep a healthy turnover of mitochondria in cells, fails due to the expression of mHTT.

4.2.1 Impact of mHTT on Cargo Recognition

Despite the fact autophagosomes are formed at comparable or even higher levels in HD cells and are sufficiently degraded by lysosomes, the degradation of the cargo, in this case mitochondria, is far more than inefficient. Therefore, it was suggested that mitophagy may be hindered at the point of distribution of damaged mitochondria or other autophagic cargo to places where autophagosomes are being formed. Although it is still not evident, what is the reason for the dysfunctional recognition of marked mitochondria as a mitophagy substrate, which should be engulfed by the autophagosome, there are suggested mechanisms that may allow us to further understand this issue.

The p62/SQSTM1 is a protein providing recognition of protein aggregates and mitochondria by the autophagy machinery thanks to its polyubiquitin binding ability through the ubiquitin-associated (UBA) domain. It links ubiquitinated proteins to the LC3 labelled isolation membrane (Donaldson et al., 2003). In fact, there was lesser colocalization of damaged organelles, such as depolarized mitochondria, and autophagic marker, the LC3, in both neuronal and non-neuronal HD cells. An *in vitro* fusion assay of LC3 marked autophagosomes and LAMP-2B marked lysosomes exhibited comparable fusion processes in ^{18}Q-HTT and ^{111}Q-HTT cells, although the homotypic fusion of autophagosomes was 33% higher in ^{111}Q-HTT cells (Martinez-Vicente et al., 2010). Taking these facts into consideration, we have been provided evidence that mitophagy is most probably blocked at the point of cargo recognition.

A study of knock-in mouse embryonic fibroblasts (MEFs) with 18 or 111 CAG introduced into the exon-1 of the mouse HTT gene showed extraordinary interaction between the ^{111}Q-HTT and p62. An immunoblot assay displaying a distribution of HTT and p62 in autophagic vacuoles showed higher presence of mHTT at the autophagosomal membrane than that of the wild-type HTT. In addition, normalized levels of p62 bound to ^{111}Q-HTT autophagic vacuoles were significantly higher than the levels in ^{18}Q-HTT mice (Martinez-Vicente et al., 2010). Additionally, p62 labelled nuclear inclusions showed co-localization with MW8 stained mHTT in HD$^{Q175/Q175}$ mice (Vodicka et al., 2014). It is possible that this unusual interaction of p62 and mHTT may be the cause of unsuccessful cargo sequestration and thus accumulation of damaged mitochondria in HD cells, because of the key role of

p62 in this process. A decrease of HTT amount in cells leads to lowered interaction between p62 and LC3 and moreover prevents p62 from binding to proteins with lysin-63-linked ubiquitin chains, which are primarily considered a substrate for autophagy. However, the depletion of HTT does not lead to these effects in the proteasomal degradation pathway. Bearing these facts in mind, HTT could act as a scaffold in mitophagy. Further observations of HTT interactions revealed its ability to associate with another autophagy machinery component, the ULK1 kinase, an inseparable factor for autophagy induction. HTT competes with mTORC1 to form the ULK1-HTT complex, which in contrast to ULK1-mTORC1 does not influence the kinase activity of ULK1, which is inhibited by mTORC1. HTT interacts with ULK1 only under conditions initiating selective autophagy such as mitophagy, not when undergoing nutrient deprivation (Rui et al., 2015).

4.2.2 Mitophagy as a Feasible Therapeutic Target

Given the fact that impaired mitophagy along with the related accumulation of damaged mitochondria remarkably contribute to the pathogenesis of HD and other neurodegenerative diseases, there is now more focus in the pharmaceutical field on targeting mitophagy as a possible therapeutic target. Although the best possible approach in this case would be gene therapy, simple replacement of non-functional genes is surrounded by ethical questions and other issues.

4.2.3 PINK1 Regulation

Usage of the *Drosophila* model system showed that direct activation of PINK1 could increase the rate of mitophagy. Electron micrographs of *Drosophila* non-apoptotic photoreceptor neurons showed that two morphologically distinct types of mitochondria were present: common round-shaped mitochondria and bent mitochondria that were bulking into ring-like structures, often enclosing cytosolic components like pigment granules. Such mitochondria were notably similar to spheroid mitochondria described in cells where mitophagy was compromised, thus implicating a mitophagy block in the *Drosophila* cells (Ding et al., 2012). This raised the question whether the overexpression of PINK1 ($PINK1^{OE}$) could rescue mitophagy. Quantitative analysis of the photoreceptors expressing mHTT indicated smaller size and higher numbers of non-spheroid mitochondria, suggesting their fragmentation. Additionally, those mitochondria were more likely to cluster together in HD flies. $PINK1^{OE}$ prevented photoreceptor depletion in flies that were 1 day old and expressing mHTT. The percentage of intact ommatidia in $PINK^{OE}$ receptors was 21% higher in HD flies. There was no detected effect of $PINK1^{OE}$ in control flies. Moreover, premature death typical of HD flies was prevented, with the mean and the median survival prolonged by 40%. This effect, however, was not observed in control flies. Furthermore, mitochondrial functions such as ATP production were rescued in $PINK1^{OE}$ HD flies (Khalil et al., 2015). To support evidence on PINK1 mediated neuroprotection, homozygous PINK1 loss-of-function allele (PINK1 B9) was introduced into the HD fly genome, producing flies with shortened lifespan (Park et al., 2006). $PINK^{B9}$ heterozygous HD flies did not show any changes in their lifespan. It was also shown that PINK1 neuroprotection was Parkin dependent, as Parkin loss-of-function allele abrogated PINK1

neuroprotection, although Parkin itself was not competent enough to act against mHTT toxicity. Overexpression of PINK1 in HDhQ7 and HDhQ111 mice led to a finding that PINK1 is capable enough to ameliorate mitochondrial turnover even in mice cells expressing mHTT (Khalil et al., 2015).

Enhancing PINK1 activity was suggested to be an efficient therapeutic strategy for PD. As the way through which the PINK1 enhanced activity is supposed to help in PD works on the basis of promoting mitophagy, we can propose that the same enhancing activity could also improve mitophagy in HD. Kinases can usually be pharmacologically activated via a small molecule binding into allosteric regulatory sites. Unfortunately, PINK1 does not offer any site for binding small molecules. On the other hand, PINK1 can utilize different substrates other than only ATP, which was discovered thanks to the studies of four PINK1 related diseases caused by insertions in the kinase domain that provides most of the interactions with the adenine involved in the ATP molecule. What is applicable in this case is the neo-substrate N^6 furfuryl ATP (KTP). KTP has even higher catalytic ability than ATP and furthermore its precursor, kinetin, can be consumed by cells and rebuilt it into KTP, which then enhances PINK1 activity, which results in the promotion of PINK1-dependent processes such as mitophagy (Hertz et al., 2013).

4.2.4 p62/SQSTSM1 Regulation

As the impairment of mitochondria is linked to most neurodegenerative diseases, recent research of compounds able to improve mitophagy attracted a lot of interest. However, there is no compound applicable *in vivo* so far. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and oligomycin are compounds applied to induce mitophagy *in vitro*. When used *in vivo*, these compounds appeared to be highly toxic (Maro et al., 1982; Padman et al., 2013).

So far the most promising target for inducing mitophagy in HD is the p62/SQSTM1 dependent recruitment of mitochondria to autophagosomal membrane. The expression of the p62 is to a certain degree facilitated by the nuclear factor erythroid 2-related factor 2 (Nrf2). The Nrf2 is mostly induced in order to adapt to oxidative stress, which is the reason why the affected genes, including the p62, have the antioxidant response element (ARE) present in their promoter regions (Kensler et al., 2007; Rushmore et al., 1991). Hence, compounds inducing Nrf2 activity can also increase the rate of p62 expression (Jain et al., 2010). In organisms, Nrf2 is activated by the interaction of pro-oxidants and electrophiles with the Nrf2 regulator, the Kelch-like ECH-associated protein 1 (Keap1). Several electrophilic natural compounds such as sulforaphane were described to up-regulate Nrf2. Sulforaphane, an isothiocyanate compound, alters functionally important cystein residues in Keap1 via oxidation or covalent modification (Dinkova-Kostova et al., 2002). These alterations lead to conformational changes which disable the subsequent ubiquitination of Nrf2. This impairment results in higher concentrations of Nrf2 and its translocation into the nucleus. After it reaches the nucleus, Nrf2 interacts with small Maf proteins and thereby initiates the transcription of genes that contain ARE in their promoter (Katsuoka et

al., 2005). Sulforaphane proved its relevance in preclinical studies of disease prevention as a protective agent against carcinogens and oxidizing substances (Kensler et al., 2007). Nevertheless, it shows nonspecific cysteine interactions with other proteins, thus gaining toxic character. Regarding the toxicity of sulforaphane, the actual compound activating ARE genes expression in an Nrf2-dependent manner that would have the covalent binding motif absent could still enhance the expression without cytotoxic effects. Based on this presumption, the p62-mediated mitophagy inducer (PMI) was developed. The results of *in vitro* testing show that PMI does not appear to be toxic at the concentrations at which sulforaphan is. The mitophagy activation provided by this compound seems to occur autonomously, apart from Parkin recruitment, and is not connected with mitochondrial membrane potential dissipation (East et al., 2014).

4.3 Methods for Monitoring Mitophagy

When monitoring mitophagy as well as any other organelle-selective type of autophagy, a number of analyses need to be performed. First of all, elevated levels of mitochondria-positive autophagosomes need to be observed. Second of all, the rates of mitochondrial degradation need to be consistent with the maturation of mitochondria-associated autophagosomes. In addition, the maturation has to be susceptible to autophagy-specific and lysosomal degradation-specific inhibitors. Finally, it is necessary to prove whether the degradation of mitochondria occurs as a result of mitophagy or due to a higher rate of mitochondrial degradation caused by nonselective autophagy (Klionsky et al., 2016).

Electron microscopy (EM) belongs to the most commonly used imaging methods of studying mitophagy. It provides the opportunity to visualize colocalization of individual mitochondria with autophagosomes and lysosomes. However, EM is not quantitative and requires advanced observation skills. In this case, fluorescent microscopy would be more useful as it does not place such high demands on observation skills and results can be quantified more easily. To show the colocalization of mitochondria and autophagosomes, a combination of MitoTracker and (GFP)-LC3 is often used. This combination enables the observation of mitochondria and LC3-positive autophagosomes, but is not suitable for observing mitochondrial degradation. However, MitoTracker staining results can easily be misinterpreted, as damaged mitochondria might not have been stained, due to the fact that MitoTracker is sensitive to mitochondrial membrane potential dissipation. This could be solved by staining mitochondria before applying any mitophagic initiator, i.e. the mitochondrial uncoupler FCCP. Labelling OMM proteins could also be misleading, as they are often degraded by proteasome. Recently, assays such as MitoTimer, mt-Keima, and mito-QC were developed to monitor mitophagy, even *in vivo*. Mito-QC, the most advantageous assay of these three, is a pH dependent tandem fluorescent tag that targets the MTS of the OMM proteins FIS1. Mito-Qc labelled mitochondria display green and red fluorescence, which is quenched after the delivery of mitochondria to lysosome. Unlike mt-Keima, mito-QC allows fixation if the fixative has neutral pH (Williams et al., 2017). The degradation of mitochondrial proteins can be measured by Western blot. Although Parkin ubiquitinates a variety of

OMM proteins such as voltage-dependent anion-selective channel 1 (VDAC1), MFN1/2 and TOM20, it is not recommended to measure their degradation, as they might have been degraded by the proteasome, as mentioned above. Moreover, the degradation could be measured as the number of mitochondria that remained undamaged. Flow cytometry using MitoTracker labelling could serve for this purpose (Klionsky et al., 2016).

5 Conclusion

Mitochondrial impairment and accumulation of mutated and misfolded proteins is a prominent hallmark of many neurodegenerative diseases. Dismissal of misfolded proteins through autophagy and other cellular mechanisms as well as dismissal of damaged mitochondria via mitophagy are essential for the preservation of cellular homeostasis.

It was implied that the gain of toxicity of HTT is not caused by the absence of any of its many functions such as apoptosis prevention and downregulation of PCGα-1. Although the role of these functions is essential, the gain of toxic function corresponds with the expansion of CAG repeats in HTT gene.

HTT associates with ULK1, an important autophagy initiator. In addition, it was suggested that HTT might serve as a scaffold which would mediate selective autophagy via interacting with p62. In HD, mHTT abnormally interacts with p62, which results in a failure of targeting mitochondria to autophagosome and an overall disruption of mitophagy.

Although $PINK^{OE}$ is a possible approach in this case and the OE led to elevated mitophagic levels in *Drosophila melanogaster*, inducing mitophagy at the point of cargo sequestration seems to be more efficient. Expression of p62/SQSTM1 can be modulated through the stimulation of the Nrf2 transcription factor. So far, only few electrophilic compounds have been described to upregulate the Nrf2-dependent expression of ARE genes like p62/SQSTM1. One of these compounds, sulforaphane, showed mediated protection against carcinogens and oxidizing substances in preclinical studies. However, sulforaphane showed to be toxic due to its nonspecific cysteine bonding to other proteins. Because of its toxic character, sulforaphane was replaced with a new compound, the PMI. PMI has not shown any toxicity in *in vitro* testing so far.

Currently, there is no compound capable of mitophagy induction *in vivo*, nor is there a cure for HD. Successful development of such a compound thus requires further research.

6 References

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